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# Adult Telomerase Positive Stem Cells: Generation of Cell-Specific Exosome-Conditioned Medium, Repetitive Single Cell Clonogenic Analysis and Genomic Labeling

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## Abstract

The adult human body is composed of trillions and trillions of cells. These cells can be divided into three categories: telomerase negative differentiated cells and progenitor cells, and telomerase positive stem cells (aTPSCs). The aTPSCs are uniquely different from differentiated cells and progenitor cells, due mainly to not conforming to standard tissue culture practices, and to their limited number in the body. Therefore, special technologies were developed to examine aTPSCs both in vitro and for their use in preclinical disease models in vivo. This is the fourth in a series of research/review articles, explaining in detail, the special technologies (e.g., rationale, standardization, reagents, manufacturers, step-by-step instructions, expected results via text, tables, and figures) that were developed. The first report detailed an introduction to aTPSCs and their location in the body. The second report detailed the isolation of mixed cell populations from solid tissue biopsy specimens and the technologies developed to optimize the viability of the aTPSCs during their isolation, plating, and propagation in vitro. The third detailed technologies to separate aTPSCs using differential cryopreservation, as well as segregating the aTPSCs into individual populations based on unique cell surface markers and cell sorting. This fourth report describes detailed technologies to obtain purified populations of individual categories of aTPSCs using repetitive single cell clonogenic analysis with cell-specific exosome-conditioned medium and genomic labeling the clonal populations to track them in vitro and in vivo.

**Keywords:** Differentiated Cells; Progenitor Cells; Stem Cells; Telomerase; Positive; Negative; Cloning; Exosomes; Beta-Galactosidase

## 1. Introduction

The adult human body is composed of trillions and trillions of cells. These cells can be subdivided into three categories based on their inherent activities, e.g., differentiated cells, progenitor cells, and healing stem cells (endogenous adult telomerase positive stem cells, aTPSCs) [1]. The 220+ differentiated cells comprise approximately 40% of the cell types in the body. Differentiated cells are composed of telomerase negative cells with a defined lifespan, e.g., 6-8 population doublings in animals [2] and 50-70 population doublings in humans [3]. Differentiated cells are divided into two categories, parenchyma and stroma. The parenchyma is the actively functioning cell within an organ or tissue and the stroma is its supportive connective tissue framework. Examples of functional differentiated cells are signal transmitting

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neurons [4,5], hormonal secreting pancreatic islet cells [6-8], contracting skeletal muscle cells [9-11], and detoxifying hepatocytes [12-14].

The 230+ progenitor cells compose approximately 59% of the cells of the body and are the immediate precursor cells to the differentiated cells. Similar to differentiated cells, they are also telomerase negative and have defined lifespans of 6-8 for rodents (Rohme) and 50-70 for humans (Hayflick). Progenitor cells are committed to form specific cell types. There are four subcategories of progenitor cells, e.g., multipotent, tripotent, bipotent, and unipotent [15]. An example of a multipotent progenitor cell is the "hematopoietic stem cell" [16-18]. It will form 18+ cell types all within the hematopoietic lineage. An example of a tripotent progenitor cell is the "(tripotent) mesenchymal stem cell". It will form three cell types, e.g., fat (unilocular white adipose cells), cartilage (hyaline cartilage), and bone (intramembranous bone) [15,19-21]. An example of a bipotent progenitor cell is the adipo-fibroblast. It will form only two cell types: unilocular white fat cells and fibroblasts/fibrocytes [22,23]. Examples of unipotent progenitor cells are neuroblasts forming neurons of the CNS, cardiac myoblasts forming cardiac myocytes of the heart, and pneumoblasts forming pneumocytes (alveolar cells) lining the respiratory passages within the lungs [24].

Healing stem cells (aTPSCs) comprise approximately 1% of the ~450+ cell types (~220+ differentiated functional cells and ~230+ maintenance progenitor cells) of the body. While small in number with respect to the surface area/volume ratio of an individual they are telomerase positive. Therefore, they exhibit essentially unlimited proliferation potential as long as they remain undifferentiated [25]. There are three major categories of healing stem cells. The first major category of the endogenous adult telomerase positive stem cells is the 0.1 to 2-micron totipotent stem cells (TSCs), and will form the same cell types as a four cell-stage blastomere, e.g., all somatic cells of the body, gender-specific gametes, the nucleus pulposus of the intervertebral disc (the only functional adult derivative of the notochord), and cells/tissues of the placenta. The second major category of aTPSCs are the pluripotent stem cells (PSCs) which will form the same cell types as the inner cell mass, e.g., all somatic cells of the body, but not the germ cells, the nucleus pulposus of the intervertebral disc, or placental tissues. The PSCs are composed of multiple subpopulations of cells in transition, e.g., >2 to <4-micron halo-like stem cells (HLSCs), 4 to <6-micron corona-like stem cells (CLSCs), 6-8-micron pluripotent stem cells (PSCs), and >8 to <10-micron germ layer lineage stem cell (a divisional transitional stem cell). The third major category of aTPSCs are three populations of 10-12-micron germ layer lineage stem cells. Ectodermal stem cells (EctoSCs) will form all somatic cell types within the ectodermal germ layer lineage (objectively identified 15 separate cell types to date based on availability of antibodies to phenotypic expression makers); mesodermal stem cells (MesoSCs) will form all somatic cell types within the mesodermal germ layer lineage (objectively identified 37 separate cell types to date), and endodermal stem cells (EndoSCs) forming all somatic cell types within the ectodermal germ layer lineage (objectively identified 14 separate cell types to date), (Table 1, Figs. 1-3) [15,26].

**Table 1** Attributes of Endogenous Adult Telomerase Positive Healing Stem Cells

| **Attributes** | **TSCs[1]** | **HLSCs[2]** | **CLSCs[3]** | **PSCs[4]** | **GLSCs[5]** | **EctoSCs[6]** | **MesoSCs[7]** | **EndoSCs[8]** |
|---|---|---|---|---|---|---|---|---|
| **Size, microns** | **0.1-2.0** | **>2-4** | **>4-<6** | **6-8** | **>8-<10** | **10-12** | **10-12** | **10-12** |
| 0.4% Trypan blue | Entire Cell Positive[9] | Halo[10] Positive, Negative | Corona[11], Positive Negative | Entire Cell Negative[12] | Entire Cell Negative | Entire Cell Negative | Entire Cell Negative | Entire Cell Negative |
| Cell Surf Markers Animals | CEA-CAM-1[13] | CEA-CAM-1$^{high}$ SSEA-4$^{low}$ | CEA-CAM-1$^{low}$ SSEA-4$^{high}$ | SSEA-4[14] | SSEA-4, Thy-1[15] | Thy-1 | Thy-1 | Thy-1 |
| Cell Surf Markers Human | CD66e[16] | CD66e$^{high}$ CD10$^{low}$ | CD66e$^{low}$ CD10$^{high}$ | CD10[17] | CD10 CD90[18] | CD56, CD90 MHC-1[19] | CD13, CD90 MHC-1 | CD90 MHC-1 |
| Expressed Genes | Telom + Bcl-2+ Nanog + Nanos + CXCR4 + | Telom + NYD | Telom + NYD | Telom + Oct-3/4 + Sonic-Hedge Hog + | Telom + NYD | Telom + NYD | Telom + NYD | Telom + NYD |

| Culture Conditions | Substrate Adhesion | Substrate Adhesion | Substrate Adhesion | Substrate Adhesion | Substrate Adhesion | Substrate Adhesion | Substrate Adhesion | Substrate Adhesion |
|---|---|---|---|---|---|---|---|---|
| Differentia Capabilities [22] | Somatic Cells, Gametes, NP of IVD, Placenta | Somatic Cells Only[24] | Somatic Cells only | Somatic Cells only | Somatic Cells only | Ectoderm Lineage Only[25] | Mesoderm Lineage Only[26] | Endo-derm Lineage Only[27] |
| Maximum Proliferation To Date | >300 Rat Popula-tion Doublings | >300 Rat Popula-tion Doublings | >300 Rat Popula-tion Doublings | >400 Rat Popula-tion Doublings | >400 Rat Popula-tion Doublings | >400 Rat Popula-tion Doublings | >690 Human Population Doublings | >400 Rat Popula-tion Doublings |
| Doubling Time | 12-14 hours | 13-15 hours | 13-15 hours | 14-16 hours | 16-18 hours | 18-24 hours | 18-24 hours | 18-24 hours |

Table 1. TSCs[1], totipotent stem cells; HLSCs[2], halo-like stem cells; CLSCs[3], corona-like stem cells; PSCs[4], GLSCs[5], germ layer lineage stem cells; EctoSCs[6], ectodermal stem cells; MesoSCs[7], mesodermal stem cells; EndoSCs[8], endodermal stem cells; entire cell demonstrating Positive[9] staining for 0.4% Trypan blue; Halo[10], complete peripheral rim of positive trypan blue staining with central area of cell negative for Trypan blue staining; Corona[11], crown of positive Trypan blue staining, remainder of cell is negative for Trypan blue staining; entire cell demonstrating Negative[13]staining for 0.4% Trypan Blue; CEA-CAM-1[13], carcinoembryonic antigen-cell adhesion molecule-1; SSEA-4[14], stage-specific embryonic antigen-4; Thy-1[15], N-glycosylated glycophosphatidylinositol (=CD90); CD66e[16], carcinoembryonic antigen; CD10[17], common acute lymphoblastic leukemia antigen (CALLA); CD90[18], N-glycosylated glycophosphatidylinositol (=Thy-1); MHC-1[19], self-recognition molecule Major Histocompatibility Complex-Class-1; Suspension[20], cells grow in suspension culture only; Substrate Adhesion[21], only grows attached to a substrate; Differentia Capabilities[22], differentiation capabilities; All Cells[23], will form all somatic cells of the body, the gametes (spermatogonia and oogonia), and the nucleus pulposus of the intervertebral disc (the only tissue derived from the notochord in adults); Somatic Cells Only[24], will only form somatic cells of the body, will not form gametes, will not form nucleus pulposus of the intervertebral disc; Ecto Lineage Only[25], will only form cells of the ectodermal germ layer lineage, will NOT form cells of either the mesodermal or the endodermal germ layer linages; Meso Lineage Only[26], will only form cells of the mesodermal germ layer lineage, will NOT form cells of either the ectodermal or the endodermal germ cell lineages; Endo Lineage Only[27], will only form cells of the endodermal germ layer lineage, will NOT form cells of either to ectodermal or mesodermal germ layer lineages; NYD[28] , not yet determined [26]. Reprinted with permission from Young HE, Speight MO. Characterization of endogenous telomerase-positive stem cells for regenerative medicine, a review. Stem Cell Regen Med 2020; 4(2):1-14.

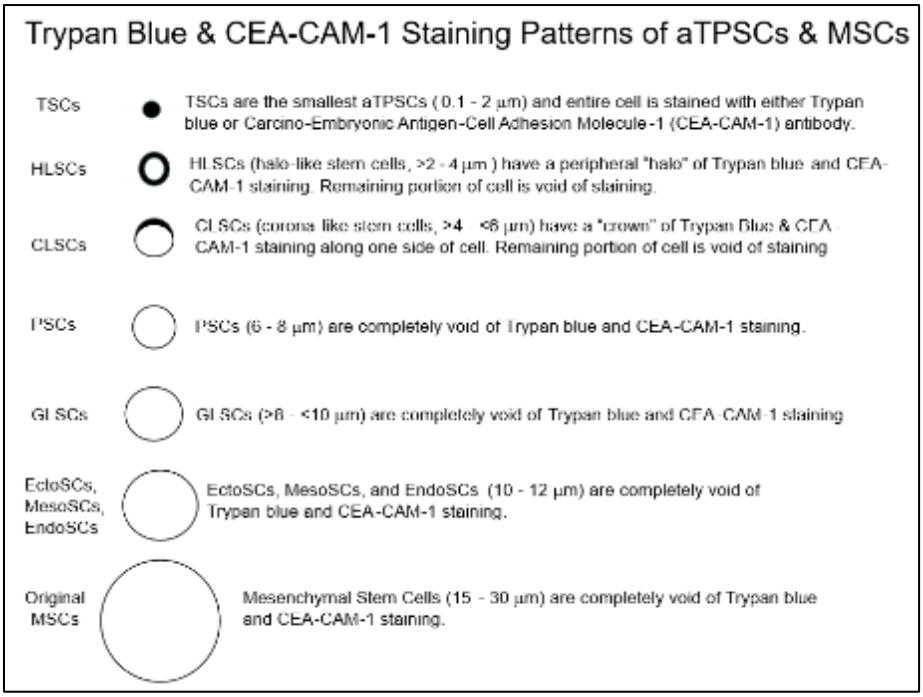


**Figure 1** Diagrammatic representation of size comparison and visual staining patterns viewed by brightfield microscopy of aTPSCs (e.g., TSCs, HLSCs, CLSCs, PSCs, GLSCs, EctoSCs, MesoSCs, and EndoSCs) and Progenitor Mesenchymal Stem Cells (MSCs) when stained with either 0.4% Trypan Blue or Carcino-Embryonic Antigen-Cell Adhesion Molecule-1 (CEA-CAM-1) antibody [27]. Reprinted with permission from Young HE. Adult telomerase positive stem cells: isolation, plating, and propagation. https://doi.org/10.30754/gscarr.2025.25.2.0355 (In press)

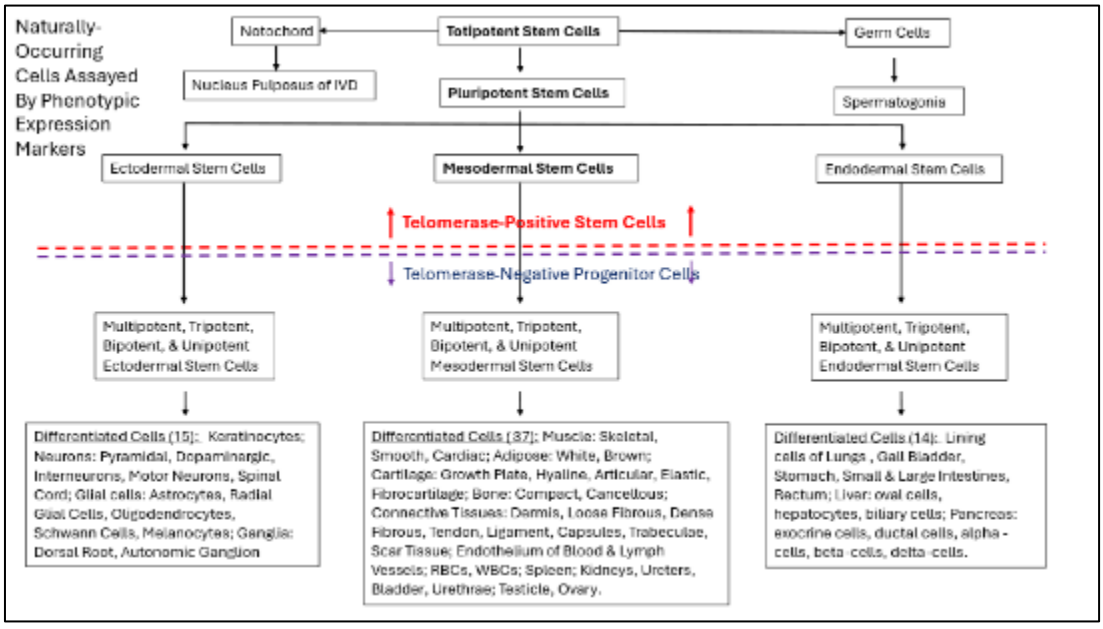


**Figure 2** Identification of eight categories of aTPSCs and their unidirectional differentiation potentials into telomerase negative progenitor cells and telomerase negative differentiated cells using specific objective assays of cell surface markers and inherent phenotypic expression markers. Unidirectional differentiation of primitive undifferentiated telomerase positive totipotent stem cells, through subsequent differentiation steps, i.e., telomerase positive pluripotent stem cells, telomerase positive ectodermal stem cells, telomerase positive mesodermal stem cells, and telomerase positive endodermal stem cells, to lose the telomerase enzyme and become telomerase negative multipotent, tripotent, bipotent, and unipotent progenitor cells, which then differentiate into terminally differentiated adult cells [15]. Reprinted with permission from Young HE, Speight MO. Characterization of endogenous telomerase-positive stem cells for regenerative medicine, a review. Stem Cell Regen Med 2020; 4(2):1-14

Healing stem cells were discovered in 1975 residing within the connective tissue stroma of adult terrestrial salamanders undergoing complete limb regeneration [28,29]. Healing stem cells (aTPSCs) were shown to regenerate all damaged or lost tissues of an amputated limb, thereby restoring histoarchitecture and function to the limb [30-33]. Since 1975, healing stem cells have been extensively characterized utilizing multiple parameters, e.g., species, location within the tissues, size, staining characteristics, cell surface markers, isolation, plating, propagation, release, cryopreservation, cell surface markers, and cell sorting [15]. Telomerase positive 0.1-2-micron TSCs and 6-8-micron cells PSCs are seen as early as the 8-cell stage embryo (Fig. 4) [34-36]

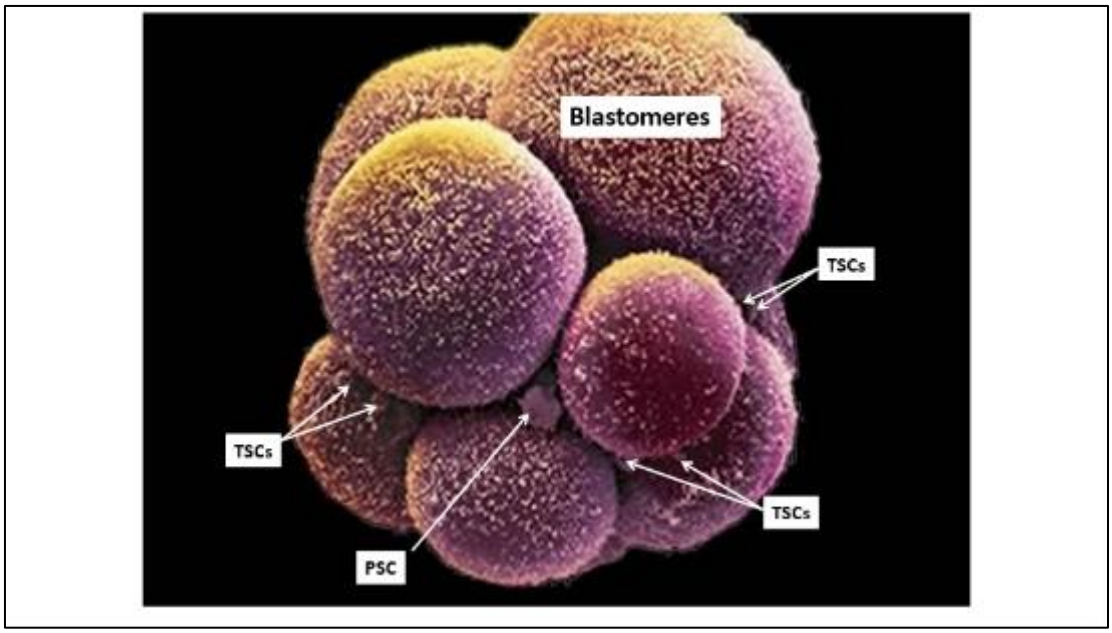


**Figure 3** Scanning electron micrograph of a male embryo, somewhere between 8-cell stage and morula. Note presence of three pairs of totipotent stem cells (TSCs) and one pluripotent stem cell (PSC). Size approximation same as seen with flow cytometry (TSCs are 0.1 to 2 microns and PSC are 6 to 8 microns in size) [34]. Reprinted with permission from Young HE, Black AC. Pluripotent Stem Cells, Endogenous versus Reprogrammed, a Review. MOJ Orthop Rheumatol. 2014; 1(4): 00019

**Table 2** Comparison / Contrast of Functional Cells, Maintenance Cells, and Healing Cells

| **Attributes** | **Differentiated Cells** | **Progenitor Cells** | **Healing Cells** | **Healing Cells** | **Healing Cells** |
|---|---|---|---|---|---|
| | | | EctoSCs<br>MesoSCs<br>EndoSCs | PSCs | TSCs |
| Adults %s | 40 | 59 | 0.9 | 0.09 | 0.01 |
| Telomerase Enzyme | Absent | Absent | Present | Present | Present |
| Location | Throughout the body | Adjacent to Differentiated Cells | Connective Tissues (CT)<br>Niches | CT<br>Niches | CT<br>Niches |
| Age Range | Newborn to Geriatric | Newborn to Geriatric | Newborn to Geriatric | Embryonic to Geriatric | Embryonic to Geriatric |
| Numbers with Aging | Decline with age | Decline with age | Remain Constant | Remain Constant | Remain Constant |
| Native Naïve State | NA | Dormant, | Dormant, | Dormant, | Dormant, |

| | | | | | |
|---|---|---|---|---|---|
| | | Quiescent, Hibernating | Quiescent, Hibernating | Quiescent, Hibernating | Quiescent, Hibernating |
| Teratoma Formation | Absent | Absent | Absent | Absent | Absent |
| Growth In vitro | Single Layer Confluent | Single Layer Confluent | Single Layer Confluent | Multi-Layered Confluent | Multi-Layered Confluent |
| Respond to Inhibitory Factors | Yes | Yes | Yes | Yes | Yes |
| Respond to Proliferative Factors | Yes | Yes | Yes | Yes | Yes |
| Proliferation Potential | 50-70 PDs in Humans 6-8 PDs Rodents | 50-70 PDs in Humans 6-8 PDs Rodents | Unlimited Humans & Rodents | Unlimited Humans & Rodents | Unlimited Humans & Rodents |
| Respond to Progression Factors | NA | Yes | No | No | No |
| Respond to Inductive Factors | No | Only in Committed Lineage | Yes | Yes | Yes |
| Cell Types Formed | NA | Only in Committed Lineage | Ectodermal Mesodermal Endodermal | All Somatic Cells | All Somatic Cells + Gametes + NP of IVD + Placenta |
| Time Period Fresh Isolate to In Vivo Use | 4 hours | 4 hours | 4 Hours | 4 hours | 4 hours |
| Time Period Fresh Isolate to Ex Vivo Use | 1-24 hours | 6-8 hours | 24 hours | 24 hours | 24 hours |
| Treatment Number Potential | Thousands | Millions | Billions | Billions to Trillions | Billions to Trillions |
| Express Self-Recognition Molecules | Yes | Yes | Yes | No | No |
| Induce Graft vs Host Disease | Yes | Yes | Yes | No | No |
| Immuno-Protected | No | No | No | Yes | Yes |
| Autologous Treatments | Yes | Yes | Yes | Yes | Yes |
| Allogeneic Treatments | No Induces GvHD[1] | No Induces GvHD | No Induces GvHD | Yes | Yes |

Table 2. Attributes of functional differentiated cells, maintenance progenitor cells, and healing stem cells. [1]GvHD, Graft versus host disease. If recipient has competent immune system, donor cells will be destroyed. If recipient has compromised immune system, donor cells will try to kill the donor [15]. Reprinted with permission from Young HE, Speight MO. Characterization of endogenous telomerase-positive stem cells for regenerative medicine, a review. Stem Cell Regen Med 2020; 4(2):1-14.

Due to their rarity and their uniqueness in not conforming to standard tissue culture practices [37], special methodologies were developed to examine endogenous aTPSCs both in vitro and for their use in vivo. Previous reports have been published 1. introducing aTPSCs and their location in the body [38]; 2. their isolation, plating, and propagation [27]; 3. differential cryopreservation, cell surface markers, and cell sorting [39]; demonstrating their in vitro characterization [15,25,34]; in vivo use in IACUC-approved pre-clinical animal models of disease [40-43]; and IRB-approved compassionate use in vivo in human clinical studies and trials of diseases [42-58]. This is the fourth in a series of articles, explaining in step-by-step detail, the methodologies (e.g., rationale, standardization, reagents, manufacturers, catalog numbers, step-by-step instructions, expected results via text, tables, and figures) that were developed for in vitro characterization and in vivo use. The current report describes the methodologies developed to generate cell-specific exosome-conditioned medium, perform repetitive single cell clonogenic analysis using the exosome-conditioned medium, and genomically labeling the cells for in vitro and in vivo analyses.

The **hypothesis** tested was that individual populations of aTPSCs and MSCs could be cloned from single cells.

## 2. Materials and methods

### 2.1. Materials

*2.1.1. Reagents: [23]*

Species-specific buffers:

- Avians – Tyrode's balanced salt solution, #T-2145 (Sigma)
- Avian Ca+2, Mg+2-free – Tyrode's balanced salt solution, #T-2145 (Sigma)
- Non-human mammals – Phosphate Buffered Saline, PBS (Sigma)
- Non-human mammals – Ca+2, Mg+2-free Phosphate Buffered Saline, PBS (Sigma)
- Humans – Dulbecco's Phosphate-Buffered Saline (10X) #310-4080AJ (GIBCO)
- Humans – Dulbecco's Phosphate-Buffered Saline (10X) #310-4080AJ (GIBCO)

Medium

- Eagle's Minimal Essential Medium with Earle's salts, #410-1100EB, GIBCO
- OptiMEM + GlutaMax, GIBCO
- Heat Inactivated Serum, Atlas Biologicals
- HS7 Horse Serum, #H7889, Sigma
- Antibiotic, Penicillin/Streptomycin, #600-514AG, GIBCO

### 2.2. Methods

*2.2.1. Cell-specific exosome-conditioned medium*

Rationale

A key point in performing the repetitive single cell clonogenic analysis is that 5-6x cell-specific exosome-conditioned medium from the same cell is necessary to clone the cells from a single cell. The reason appears to be that aTPSCs are "socialized cells" meaning they are only "happy" and propagate if they are surrounded by many neighboring cells. To recreate many neighboring cells, we collected the conditioned medium from multiple confluent layers of cells (TSCs and PSCs) or single 100% confluent layer of EctoSCs, MesoSCs, EndoSCs, and MSCs. The tripotent-MSCs presented a problem for us since it did not conform to culturing conditions that aTPSCs had established for themselves. To maintain a culture of 95% confluent tripotent-MSCs we removed PDGF-BB from the propagation medium, which placed the cells in lag phase growth, and collected conditioned medium at designated intervals. The designated intervals were based on the color of the OptiMEM-based medium (Fig. 4). As the cells undergo physiology/metabolism the OptiMEM medium changes color from salmon to salmon-orange to orange-orange to orange-yellow to yellow-yellow to murky-yellow. The maximum conditioned medium collected before cell death occurred when the medium turned orange-yellow.

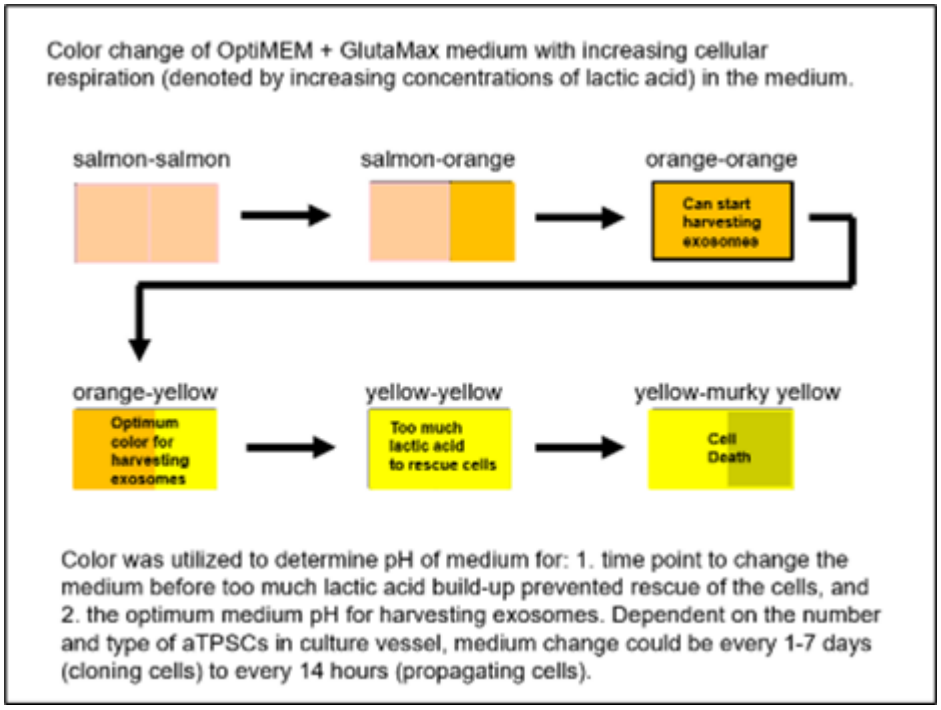


**Figure 4** Colour change of plating medium, propagation medium, cloning medium, etc., allowed the opportunity to maintain optimal growth conditions by changing medium based on the "wants" of the cells. The sequential change of colour from salmon to yellow meant an increase in lactic acid build-up in the medium. Too much lactic acid in the medium, denoted by yellow-yellow of the medium, meant that the cells could no longer be rescued by adding fresh medium. Dead cells in the medium, denoted by yellow-murky yellow colour, meant mass die off of the cells. Reprinted with permission from Young HE. Adult telomerase positive stem cells: isolation, plating, and propagation. GSC Advanced Research and Review. https://doi.org/10.30754/gscarr.2025.25.2.0355 (In press)

Procedures

To derive 5-6x exosome conditioned medium, cells purified by differential cryopreservation and cell sorting [39] were grown in complete medium (e.g., OptiMEM + GlutaMax, 10% Heat-Inactivated serum, 1% antibiotic, pH 7.4). For telomerase negative MSCs that meant that the cells were grown to 95% subconfluent density before replating. For telomerase positive EndoSCs, MesoSCs, EctoSCs, and GLSCs that meant the cells are grown to single layer confluence. For telomerase positive CLSCs, HLSCs, PSCs, and TSCs that means growing cells to multi-layered confluence. Remove medium when color shift of medium occurred from salmon-salmon to orange-yellow (Fig. 4). Remove medium, add same volume of fresh medium to 1x conditioned medium and place onto cells. When color changes from salmon-orange to orange-yellow, repeat procedure for 2x conditioned medium. Feed culture with 2x conditioned medium 1:1 with fresh medium. When medium color changes from salmon-orange to orange-yellow, remove 3x conditioned medium. And repeat procedures until 5-6x conditioned medium is generated. If medium changes from orange-yellow to yellow-yellow, the culture medium contains too much lactic acid to rescue the cells. If the medium changes from yellow-yellow to yellow-murky, there are floating dead cells. If medium is either yellow-yellow or yellow-murky, need to start again with the last successful "#-x conditioned medium". If the TSCs, HLSCs, CLSCs, and PSCs cultures form so many cell layers that feeding with fresh medium occurs every 1-2 hours, then the cells need to be replated into fresh 1% collagen-coated flasks utilizing EGTA – collagenase/dispase release and plating procedures [27]. Initial plating densities are listed [27].

Each time the cells needed to be re-plated, the numbering of the generation of exosome- conditioned medium would start at 1x. At each medium change, excess conditioned medium was frozen at -20$^{o}$C in individual tissue culture bottles labeled as to "#x-conditioned cell type medium" for particular cell. For example, at second medium change for PSCs, the bottle would be labeled "2x conditioned PSC medium". Once sufficient conditioned medium was obtained (~500-ml), the bottles of frozen, exosome-conditioned medium were thawed, and refrozen and thawed two additional times to ensure that no cells survived the freeze/thaw that could possibly contaminate the future clones. The conditioned medium was then sterile-filtered using aspiration with 0.1-micron bottle-top filters, and labeled "sterile 5-6x cell type conditioned medium".

*2.2.2. Repetitive single cell clonogenic analysis*

Rationale

There are four key points that must be adhered to for successful repetitive single cell clonogenic analysis of telomerase positive stem cells: substratum, culture medium, serum, and exosome-conditioned medium [27].

Procedure

A cryovial of purified cells was removed from freezer and placed into 37°C water bath. When medium color changed from bright yellow (frozen) to salmon colored (thawed) the cryovial was removed from the water bath. The cell suspension/DMSO mixture was added to 14-ml of complete medium, inverted 5-6 times to mix contents and centrifuged to pellet cells [27]. The supernatant was removed to bleach, and the cell pellet resuspended in 5-mililiters of 5-6x conditioned medium diluted 1:1 with fresh complete medium. Cells were counted using 0.4% sterile Trypan blue staining with a hemocytometer. Approximately 500 cells per 96-well plate were used. To 500 cells (approximating 1 cell per 5-microliters of medium), add 2.5-mililiters of 5-6x conditioned medium. Five-microliters of cell suspension was removed with a micropipettor and a drop of cell suspension was placed onto the center of each well of a 1% type-1 collagen-coated 96-well plate [Fig. 5]. As each 96-well plate was completed, the plate was covered with its lid and place into humidified, 5% CO2, 95% ambient air, 37°C incubator for 6 hours to allow attachment of cell. The plates were removed from the incubator and each well was scored for number of cells per well. GLSCs, EctoSCs, MesoSCs, EndoSCs, and MSCs divide every 24-48 hours and TSCs, PSCs, HLSCs, and CLSCs divide every 14-18 hours, a 6-hour incubation is too short a time for cell division to occur in these cells. Any well that has no cells or 2 or more cells is removed from the population. This occurred by using an indelible marker and blackening the outside bottom of the well. Add 100-microliters of 70% isopropyl alcohol to each well with blackened bottom for 10 minutes. If 70% alcohol remains in the wells for longer than 10 min with either no cells or two or more cells, the vaporization of the alcohol after placement into an incubator will kill any viable cells in adjacent wells. Therefore, the 70% alcohol is removed by aspiration and 100-microlitiers of 0.5% aqueous sterile sodium azide was added to wells with blackened well bottoms to prevent bacterial contamination. To wells without a blackened bottom, feed each well with 200-microliters of 5-6x conditioned medium. Place back into incubator. Check "clear" non-blackened wells daily. When medium changes color from salmon-orange to orange-yellow, remove 100-microliters of "spent" culture medium and feed culture with 50-microliters fresh 5-6x conditioned medium diluted 1:1 with 50-microliters of fresh complete medium. Keep a record of number of cells per well and/or percentage of cellular confluence. The maximum cellular confluence for MSCs is 95%, before replating. For GLSCs, EctoSCs, MesoSCs, EndoSCs, maximum cellular confluence is 100% before replating. And for TSCs, PSCs, HLSCs, and CLSCs, the maximum confluence percentage before replating is 600% to 800% [27].

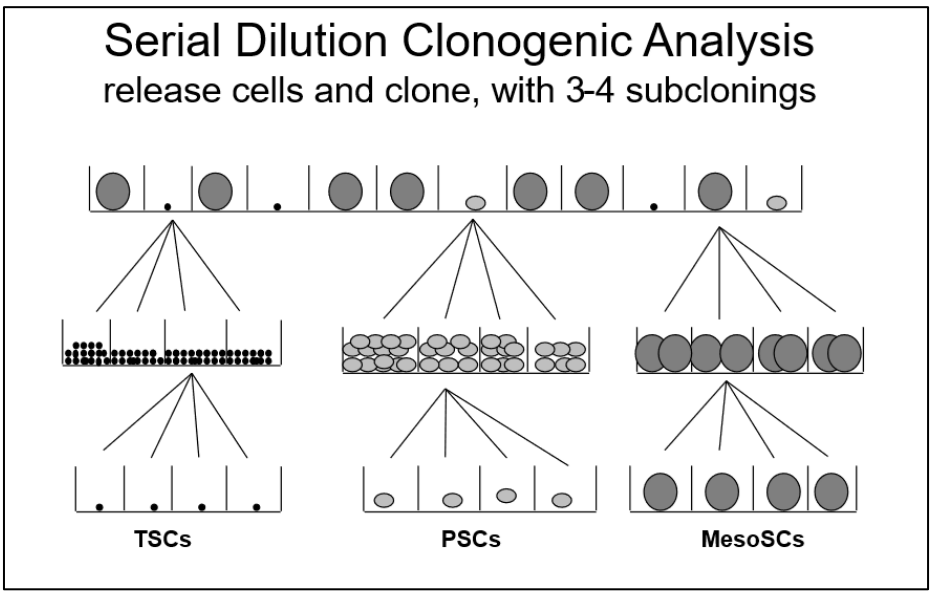


**Figure 5** Repetitive serial dilution single cell clonogenic analysis for TSCs, PSCs, and MesoSCs. Single cells plated per well of 96-well plates, grown post confluence, replated as single cells per well, grown post confluence, 3-4 times to ensure that population of cells came from a single cell

If all the cells in the well display apoptotic features, fix contents of well with 70% alcohol, then replace with 100-microliters of 0.5% aqueous sterile sodium Azide. Aqueous azide will prevent bacterial contamination in individual wells without impinging on cell viability in adjacent wells in 96-well plates. When wells reach multi-layered confluence, replate cells directly into 48-well plates. When cells reach multi-layered confluence, replate cells directly into 24 well plates. From 24-well plates, each well showing multi-layered confluent cells can be released, cells counted, 500 cells removed and diluted in 2.5-mililiters of 5-6x conditioned medium. Then 5-microliters of cell suspension plated into center of each well of fresh 96-well plate and the process repeated. This occurred 3-4 times [Fig. 5] for TSCs, HLSCs, CLSCs, PSCs, GLSCs, EctoSCs, MesoSCs, EndoSCs, and MSCs, to ensure that the clonal population was generated from a single cell. Once 3-4 re-clonings occurred, cells were propagated into T-75 flasks to expand the stem cell population and cryopreserved for future use [25].

*2.2.3. Genomic labeling*

Rationale

A genomic-labeling procedure was used to ensure that the cells could be tracked, both in vitro and after transplantation in vivo, and that the label would not decrease in intensity even after multiple cell divisions. Also, the clones were examined after beta-galactosidase labeling to ensure that the genomic labeling did not alter any of the characteristics of the clones before labeling.

Procedure

Genomic labeling of the healing stem cells (aTPSCs) was performed in collaboration with C. Duplaa, INSERM, France, so that labeled aTPSC clones could be tracked both in vitro, ex vivo, and in situ after transplantation. Fifty-six clones were generated by repetitive serial dilution clonogenic analysis. Individually, each clone was incubated with an insect Lac-Z gene for beta-Galactosidase (beta-Gal) in the presence of lipofectin. Lipofectin incorporates genetic material into the nucleus during the S and G2 phases of the cell cycle [26]. Therefore, for cells that undergo cell doubling at a comparative rapid rate, the majority of cells will incorporate the label. Greater than 95% of the aTPSCs incorporated Lac-Z into their genome. In contrast, slow cell doubling will incorporate less of the Lac-Z gene, leading to a smaller number of labeled cells. Less than 5% of the cells within the MSC clones were labeled [27].

*2.2.4. Location of genomic labeling within the cells*

Rationale

Proof was needed to ascertain that the genomic labeling of the clones would allow the tracking the cells both in vitro and after transplantation in vivo.

Procedure

Two myocardial infarction models were created in adult Sprague-Dawley rats [27]. In the first model, the apex of the heart was frozen in liquid nitrogen. Then the PSC clone Scl-40 was injected into the damaged area of the left ventricle. In the second model, the left anterior descending coronary artery (the widow maker) was transiently ligated. Then the PSC clone Scl-40 was delivered systemically by tail vein injection. After designated time periods, the animals were euthanized, tissue biopsy specimens removed, fixed, embedded, sectioned, and stained with an antibody to beta-galactosidase [27].

## 3. Results

### 3.1. Cell-specific exosome-conditioned medium

A key point in performing the repetitive single cell clonogenic analysis is that you need to use 5-6x cell-specific exosome-conditioned medium from the same cell to clone subsequent cells from a single cell (Table 3). It was noted that conditioned medium from the immediate downstream cell type could induce the immediate upstream cell type to form the downstream cell type. For example, HLSC exosome conditioned medium would induce the TSCs to form HLSCs (Table 3). However, exosome-conditioned medium from an upstream cell type would not induce a downstream cell type to dedifferentiate into an upstream cell type. Differentiation of aTPSCs was unidirectional from most primitive (TSCs) to more differentiated (progenitor cells).

**Table 3** Cell-Specific Exosome-Conditioned Medium

| **Medium (Med) Cloning** | **TSCs Med** | **HLSCs Med** | **CLSCs Med** | **PSCs Med** | **GLSCs Med** | **EctoSCs Med** | **MesoSCs Med** | **EndoSCs Med** | **MSCs Med** |
|---|---|---|---|---|---|---|---|---|---|
| TSCs | Clone | HLSCs | | | | | | | |
| HLSCs | | Clone | CLSCs | | | | | | |
| CLSCs | | | Clone | PSCs | | | | | |
| PSCs | | | | Clone | GLSCs | | | | |
| GLSCs | | | | | Clone | EctoSCs | MesoSCs | EndoSCs | |
| EctoSCs | | | | | | Clone | | | |
| MesoSCs | | | | | | | Clone | | MSCs |
| EndoSCs | | | | | | | | Clone | |
| MSCs | | | | | | | | | Clone |

Table 3. It is necessary to use conditioned medium from the same cell type to clone using repetitive serial dilution clonogenic analysis.

### 3.2. Repetitive single cell clonogenic analysis

The respective attributes of aTPSCs and MSCs clones were based on the following characterization parameters (Table 4).

**Table 4** Characterization of Cloned Cells from Rat Biopsy Specimens

| **Char[1]** | **TSCs[2]** | **HLSCs[3]** | **CLSCs[4]** | **PSCs[5]** | **GLSCs[6]** | **Ecto-SCs[7]** | **Meso-SCs[8]** | **Endo-SCs[9]** | **MSCs[10]** |
|---|---|---|---|---|---|---|---|---|---|
| Size, microns | 0.1 – 2.0 | >2 - 4 | >4 - <6 | 6-8 | 8-10 | 10-12 | 10-12 | 10-12 | 15-30 |
| Trypan Blue | Positive | Halo Positive / Remain-der Negative | Corona Positive / Remain-der Negative | All Negative | All Negative | All Negative | All Negative | All Negative | All Negative |
| Non-Human Cell Surface | CEA-CAM-1 | CEA-CAM-$1^{High}$ | CEA-CAM-$1^{Low}$ | SSEA-4 | SSEA-$4^{High}$ Thy-$1^{Low}$ | Thy-1 | Thy-1 | Thy-1 | Thy-1 |

| Markers | | SSEA-4$^{Low}$ | SSEA-4$^{High}$ | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Cryopre-servative Agent | 99-100% DMSO | 99-100% DMSO | 99-100% DMSO | 99-100% DMSO | 99-100% DMSO | 99-100% DMSO | 99-100% DMSO | 99-100% DMSO | 99-100% DMSO |
| Concen-tration of Cryo Agent | 7.5% | 7.5% | 7.5% | 7.5% | 7.5% | 7.5% | 7.5% | 7.5% | 10% |
| Optimum Number of Cells Frozen | 1-10 Billion | 1-10 Billion | 1-10 Billion | 1-10 Billion | 1-10 Million | 1-10 Million | 1-10 Million | 1-10 Million | 1-10 Million |
| Optimum Freezing Temp | -80$^{o}$C | -80$^{o}$C | -80$^{o}$C | -80$^{o}$C | -70$^{o}$C | -70$^{o}$C | -70$^{o}$C | -70$^{o}$C | -196$^{o}$C |
| Optimum Freezing Rate | Slow 1$^{o}$ Per Minute | Slow 1$^{o}$ Per Minute | Slow 1$^{o}$ Per Minute | Slow 1$^{o}$ Per Minute | Slow 1$^{o}$ Per Minute | Slow 1$^{o}$ Per Minute | Slow 1$^{o}$ Per Minute | Slow 1$^{o}$ Per Minute | Fast Flash Freeze |
| Optimum Storage Temp | -80$^{o}$C | -80$^{o}$C | -80$^{o}$C | -80$^{o}$C | -70$^{o}$C | -70$^{o}$C | -70$^{o}$C | -70$^{o}$C | -196$^{o}$C |
| Thaw Temp | 37$^{o}$C | 37$^{o}$C | 37$^{o}$C | 37$^{o}$C | 37$^{o}$C | 37$^{o}$C | 37$^{o}$C | 37$^{o}$C | 37$^{o}$C |
| % Recovery Viable Cells | >98 | >98 | >98 | >98 | >98 | >98 | >98 | >98 | >95 |
| Charac-terization of the cells | Forms All Somatic Cells of Body + GCs[23] + NP[24] of IVD[25] + Placenta | Forms All Somatic Cells of Body | Forms All Somatic Cells of Body | Forms All Somatic Cells of Body | Forms All Somatic Cells of Body | Forms All Ecto Somatic Cells of Body | Forms All Meso Somatic Cells of Body | Forms All Endo Somatic Cells of Body | Forms Fat, Cart, Bone |
| Telomerase | Positive | Positive | Positive | Positive | Positive | Positive | Positive | Positive | Negative |
| With Aging | Con-stant | Constan t | Constan t | Con-stant | Con-stant | Con-stant | Con-stant | Con-stant | De-crease |
| Viability Post Mortem | 30-40 days | >2 days | >2 days | >7 days | >5 days | >3 days | >3 days | >3 days | 1 day |
| Viability Temp[11] | 4$^{o}$C | 4$^{o}$C | 4$^{o}$C | 4$^{o}$C | 4$^{o}$C | 4$^{o}$C | 4$^{o}$C | 4$^{o}$C | 4$^{o}$C |
| Solid Tissues | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| Connective Tissue Niches | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| Isolation | | | | | | | | | |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Bone Marrow | Yes | NYD[12] | NYD | Yes | Yes | Yes | Yes | Yes | Yes |
| Isolation Adipose Tissue | Yes | NYD | NYD | Yes | Yes | Yes | Yes | Yes | Yes |
| Presence Wharton's Jelly Umbilical Cord | Yes | NYD | NYD | Yes | Yes | Yes | Yes | Yes | Yes |
| Circulating In Blood | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | No |
| Species Utilized | Avian, Mouse, Rat | Avian, Mouse, Rat | Avian, Mouse, Rat | Avian, Mouse, Rat | Avian, Mouse, Rat | Avian, Mouse, Rat | Avian, Mouse, Rat | Avian, Mouse, Rat | Avian, Mouse, Rat |
| Clones Generated Genomic-Label (□) | Scl-4□, Scl-9□ Scl-44□ | NYD | NYD | Scl-40□ | NYD | NYD | Scl-A2A2□ | NYD | Rt-MSC[13], Rt-Adip[14], Rt-Chon[15], Rt-Os[16] |
| Contact Inhibited | No | No | No | No | Yes | Yes | Yes | Yes | Yes |
| Survive Contact Inhibited | NA[17] | NA | NA | NA | Yes | Yes | Yes | Yes | No |
| Growth in Culture | Adhe-sion | Adhe-sion | Adhe-sion | Adhe-sion | Adhe-sion | Adhe-sion | Adhe-sion | Adhe-sion | Adhe-sion |
| Provide Substrate Type | Yes Collag-1[18] | Yes Collag-1 | Yes Collag-1 | Yes Collag-1 | Yes Collag-1 | Yes Collag-1 | Yes Collag-1 | Yes Collag-1 | No Bare Plastic |
| Activity in Serum-Free Defined Medium | Quies-cent | Quies-cent | Quies-cent | Quies-cent | Quies-cent | Quies-cent | Quies-cent | Quies-cent | Quies-cent |
| Activity in Complete Medium Only (Control) | Quies-cent | Quies-cent | Quies-cent | Quies-cent | Quies-cent | Quies-cent | Quies-cent | Quies-cent | Quies-cent |
| Complete Medium + Inhibitory Factors[19] | Respond Prevents Differen-tiation | Respond Prevents Differen-tiation | Respond Prevents Differen-tiation | Respond Prevents Differen-tiation | Respond Prevents Differen-tiation | Respond Prevents Differen-tiation | Respond Prevents Differen-tiation | Respond Prevents Differen-tiation | Respond Prevents Differen-tiation |
| Complete Medium + | Respond | Respond | Respond | Respond | Respond | Respond | Respond | Respond | Respond |

| Proliferation Factors[20] | Prolife-rates | Prolife-rates | Prolife-rates | Prolife-rates | Prolife-rates | Prolife-rates | Prolife-rates | Prolife-rates | Prolife-rates |
|---|---|---|---|---|---|---|---|---|---|
| Complete Medium + Progression Factors[21] | Does not Respond | Does not Respond | Does not Respond | Does not Respond | Does not Respond | Does not Respond | Does not Respond | Does not Respond | Forms Fat, Cart, Bone |
| Complete Medium + Inductive Factors[22] | Forms All Somatic Cells of Body + GCs[23] + NP[24] of IVD[25] + Placenta | Forms All Somatic Cells of Body | Forms All Somatic Cells of Body | Forms All Somatic Cells of Body | Forms All Somatic Cells of Body | Forms All Ecto Somatic Cells of Body | Forms All Meso Somatic Cells of Body | Forms All Endo Somatic Cells of Body | Forms Fat, Cart, Bone |
| Number of Cell Types Formed | 75 | 72 | 71 | 70 | 69 | 15 | 37 | 14 | 3 |
| Number of Lineages formed | 3 + Germ Cells + NP of IVD | 3 | 3 | 3 | 3 | 1 | 1 | 1 | 1 |
| Proliferation Rate | 12-14 Hours | 12-14 Hours | 12-14 Hours | 12-14 Hours | 14-18 Hours | 18-24 Hours | 18-24 Hours | 18-24 Hours | Days to Weeks |
| Population Doublings | >300 | >300 | >300 | >400 | >400 | >400 | >690 | >400 | 50-70 |
| Karyotype | Normal | NYD | NYD | Normal | NYD | Normal | Normal | Normal | Normal |
| Expressed Genes | Telom, Bcl-2, Nanog, Nanos, CXCR4 | Telom + NYD | Telom + NYD | Telom, Oct-3/4, Sonic-Hedge Hog | Telom + NYD | Telom + NYD | Telom + NYD | Telom + NYD | NYD |

Table 4. Char[1], characteristics tested; TSCs[2], Totipotent Stem Cells; HLSCs[3], Halo-like Stem Cells; CLSCs[4], Corona-Like Stem Cells; PSCs[5], Pluripotent Stem Cells; GLSCs[6], Germ Layer Lineage Stem Cells; EctoSCs[7], Ectodermal Stem Cells; MesoSCs[8]; Mesodermal Stem Cells; EndoSCs[9], Endodermal Stem Cells; MSCs[10], tripotent progenitor Mesenchymal Stem Cells; Temp[11], Temperature; NYD[12], Not Yet Determined; Rt-MSC[13], telomerase negative rat tripotent progenitor Mesenchymal Stem Cell Clone; Rt-Adipo[14], telomerase negative rat unipotent progenitor Adipoblast Clone; Rt-Chon[15], telomerase negative rat unipotent progenitor Chondroblast Clone; Rt-Os[16], telomerase negative rat unipotent progenitor Osteoblast Cell Clone; NA[17], Not Applicable; Collag-1[18], Collagen Type-1; Inhibitory Factors[19], inhibit differentiation of stem cells and progenitor cells, e.g., LIF (leukemia inhibitory factor) and ADF (anti-differentiation factor); Proliferation Factors[20], stimulate proliferation of stem cells and progenitor cells, e.g., PDGF-AA (platelet derived growth factor-AA, PDGF-AB (platelet-derived growth factor-AB), and PDGF-BB (platelet derived growth factor-BB); Progression Factors[21], accelerates phenotypic expression in lineage-committed progenitor cells no effect on stem cells, e.g., insulin, IGF-1 (insulin-like growth factor-1), IGF-2 (insulin-like growth factor-2); Inductive Factors[22], induces lineage-commitment in stem cells, e.g., recombinant proteins, morphogenic proteins derived from demineralized bone matrix, and cell-specific exosome conditioned medium; GCs[23], Germ Cells (spermatogonia, oogonia); NP[24], nucleus pulposis; IVD[25], intervertebral disc [15]. Reprinted with permission from Young HE, Speight MO. Characterization of endogenous telomerase-positive stem cells for regenerative medicine, a review. Stem Cell Regen Med 2020; 4(2):1-14.

### 3.3. Genomic labeling of clones with beta-galactosidase

Thus far, five beta-Gal labeled clones have been fully characterized, e.g., Scl-4 (TSC clone), Scl-9 (TSC clone), Scl-44 (TSC clone), Scl-40 (PSC clone), Scl-$A_2A_2$ (MesoSC clone). Additionally, four unlabeled rat-derived progenitor cell clones (Rt-MSC, Rt-Adip, Rt-Chon, and Rt-Os) were also characterized (Table 5). Their respective attributes were based on the following characterization parameters established previously (Table 4) and [15].

**Table 5** Characterization of Genomically-Labeled Cloned TSCs, PSCs, MesoSCs and Non-Labeled MSC Clones from Rat Biopsy Specimens

| **Char[1]** | **Scl-4 TSC Clone[2]** | **Scl-9 TSC Clone** | **Scl-44 TSC Clone** | **Scl-40 PSC Clone[3]** | **Scl-A2A2 MesoSC Clone[4]** | **Rt-MSC Tripotent MSC Clone[5]** | **Rt-Adip Uni-potent Fat Clone[6]** | **Rt-Chon Uni-Potent Cart Clone[7]** | **Rt-Os Uni-Potent Bone Clone[8]** |
|---|---|---|---|---|---|---|---|---|---|
| Size, microns | 0.1 – 2.0 | 0.1 – 2.0 | 0.1 – 2.0 | 6-8 | 10-12 | 15-30 | 15-30 | 15-30 | 15-30 |
| Trypan Blue | Positive | Positive | Positive | All negative | All negative | All negative | All negative | All negative | All negative |
| Telomerase | Positive | Positive | Positive | Positive | Positive | Negative | Negative | Negative | Negative |
| With Aging | Constant | Constant | Constant | Constant | Constant | Decrease | Decrease | Decrease | Decrease |
| Viability Post Mortem | 30-40 days | 30-40 days | 30-40 days | >7 days | >3 days | 1 day | 1 day | 1 day | 1 day |
| Viability Temp | 4°C | 4°C | 4°C | 4°C | 4°C | 4°C | 4°C | 4°C | 4°C |
| Solid Tissues | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| Connective Tissue Niches | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| Isolation Bone Marrow | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| Isolation Adipose Tissue | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| Isolation Wharton's Jelly - UC | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| Circulating In Blood | Yes | Yes | Yes | Yes | Yes | No | No | No | No |
| Species Utilized | Avian, Mouse, Rat | Avian, Mouse, Rat | Avian, Mouse, Rat | Avian, Mouse, Rat | Avian, Mouse, Rat | Avian, Mouse, Rat | Avian, Mouse, Rat | Avian, Mouse, Rat | Avian, Mouse, Rat |
| Genomic-Label Single Cell Clones | Scl-44b, Scl-9b Scl-4b | Scl-44b, Scl-9b Scl-4b | Scl-44b, Scl-9b Scl-4b | Scl-40b | Scl-A2A2 | Rt-MSC, Rt-Adip, Rt-Chon, Rt-Os | Rt-MSC, Rt-Adip, Rt-Chon, Rt-Os | Rt-MSC, Rt-Adip, Rt-Chon, Rt-Os | Rt-MSC, Rt-Adip, Rt-Chon, Rt-Os |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Contact Inhibited | NA | NA | NA | NA | Yes | Yes | Yes | Yes | Yes |
| Survive Contact Inhibited | NA | NA | NA | NA | Yes | No | No | No | No |
| Growth in Culture | Adhesion | Adhesion | Adhesion | Adhesion | Adhesion | Adhesion | Adhesion | Adhesion | Adhesion |
| Provide Substrate ?? | Yes Collag-1 | Yes Collag-1 | Yes Collag-1 | Yes Collag-1 | Yes Collag-1 | No | No | No | No |
| Activity in Serum-Free Defined Medium | Quies-cent | Quies-cent | Quies-cent | Quies-cent | Quies-cent | Quies-cent | Quies-cent | Quies-cent | Quies-cent |
| Complete Medium Only (Control) | Quies-cent | Quies-cent | Quies-cent | Quies-cent | Quies-cent | Quies-cent | Quies-cent | Quies-cent | Quies-cent |
| Complete Medium + Inhibitory Factor | Respond Prevents Differen-tiation | Respond Prevents Differen-tiation | Respond Prevents Differen-tiation | Respond Prevents Differen-tiation | Respond Prevents Differen-tiation | Respond Prevents Differen-tiation | Respond Prevents Differen-tiation | Respond Prevents Differen-tiation | Respond Prevents Differen-tiation |
| Complete Medium + Proliferation Factor | Respond Prolife-rates | Respond Prolife-rates | Respond Prolife-rates | Respond Prolife-rates | Respond Prolife-rates | Respond Prolife-rates | Respond Prolife-rates | Respond Prolife-rates | Respond Prolife-rates |
| Complete Medium + Progression Factor | Does not Respond | Does not Respond | Does not Respond | Does not Respond | Does not Respond | Forms Fat, Cart, Bone | Forms Fat | Forms Cart | Forms Bone |
| Complete Medium + Inductive Factors | Respond Forms:# | Respond Forms:# | Respond Forms:# | Respond Forms:# | Respond Forms:# | Respond Forms:# | Respond Forms:# | Respond Forms:# | Respond Forms:# |
| Number of Cells Formed | 75 | 75 | 75 | 70 | 37 | 3 | 1 | 1 | 1 |
| Number of Lineages Formed | 3 + Gem Cells + NP of IVD | 3 + Gem Cells + NP of IVD | 3 + Gem Cells + NP of IVD | 3 | 1 | 1 | 1 | 1 | 1 |
| Proliferation Rate | 12-14 Hours | 12-14 Hours | 12-14 Hours | 12-14 Hours | 18-24 Hours | Days to Weeks | Days to Weeks | Days to Weeks | Days to Weeks |
| Population Doublings | >300 | >300 | >300 | >400 | >690 | 50-70 | 50-70 | 50-70 | 50-70 |
| Cryopreser-vative Agent | 99-100% DMSO | 99-100% DMSO | 99-100% DMSO | 99-100% DMSO | 99-100% DMSO | 99-100% DMSO | 99-100% DMSO | 99-100% DMSO | 99-100% DMSO |
| Concentra-tion of Agent | 7.5% | 7.5% | 7.5% | 7.5% | 7.5% | 10% | 10% | 10% | 10% |

| Number of Cells Frozen | 1-10 Billion | 1-10 Billion | 1-10 Billion | 1-10 Billion | 1-10 Million | 1-10 Million | 1-10 Million | 1-10 Million | 1-10 Million |
|---|---|---|---|---|---|---|---|---|---|
| Optimum Freezing Temperature | -80°C | -80°C | -80°C | -80°C | -70°C | -196°C | -196°C | -196°C | -196°C |
| Optimum Freezing Rate | Slow 1° Per Minute | Slow 1° Per Minute | Slow 1° Per Minute | Slow 1° Per Minute | Slow 1° Per Minute | Fast Flash Freeze | Fast Flash Freeze | Fast Flash Freeze | Fast Flash Freeze |
| Optimum Storage Temperature | -80°C | -80°C | -80°C | -80°C | -70°C | -196°C | -196°C | -196°C | -196°C |
| Thaw Temperature | 37°C | 37°C | 37°C | 37°C | 37°C | 37°C | 37°C | 37°C | 37°C |
| % Recovery Viable Cells | >98 | >98 | >98 | >98 | >98 | >95 | >95 | >95 | >95 |
| Karyotype | Normal | Normal | Normal | Normal | Normal | Normal | Normal | Normal | Normal |
| Expressed Genes | Telom, Bcl-2, Nanog, Nanos, CXCR4 | Telom, Bcl-2, Nanog, Nanos, CXCR4 | Telom, Bcl-2, Nanog, Nanos, CXCR4 | Telom, Oct-3/4, Sonic-Hedge Hog | Telom + NYD | NYD | NYD | NYD | NYD |
| Non-Human Cell Surface Markers | CEA-CAM-1 | CEA-CAM-1 | CEA-CAM-1 | SSEA-4 | Thy-1 | Thy-1 | Thy-1 | Thy-1 | Thy-1 |

Table 5. Char[1], Characteristics; Scl-4 TSC Clone[2], Subclone-4 Beta-Galactosidase genomically-labeled totipotent stem cell clone; Scl-40 PSC Clone[3], Subclone-40 Beta-Galactosidase genomically-labeled pluripotent stem cell clone; Scl-A2A2b MesoSC Clone[4], Subclone-A2A2 Galactosidase genomically-labeled Mesodermal Stem Cell Clone; Rt-MSC Tripotent MSC Clone[5], Unlabeled Rat Tripotent Progenitor Mesenchymal Stem Cell Clone; Rt-Adip Unipotent Clone[6], Unlabeled Rat Unipotent Progenitor Adipogenic (fat) Clone; Rt-Chon Unipotent Clone[7], Unlabeled Rat Unipotent Progenitor Chondrogenic (cartilage) Clone; Rt-Os Unipotent Clone[8], Unlabeled Rat Unipotent Progenitor Osteogenic (bone) Clone.

### 3.4. Location of genomic labeling within the cells and implanted in tissues

In the undifferentiated state, the genomic beta-galactosidase label is located within the nucleus of the cloned cell, Scl-40 (Fig. 6A). With subsequent differentiation, the genomic label is located in the cytoplasm of the cell. Scl-40 differentiating into vasculature (Fig. 6B); into the myocardium (Fig. 6C), and into the connective tissue cardiac skeleton Fig. 6D).

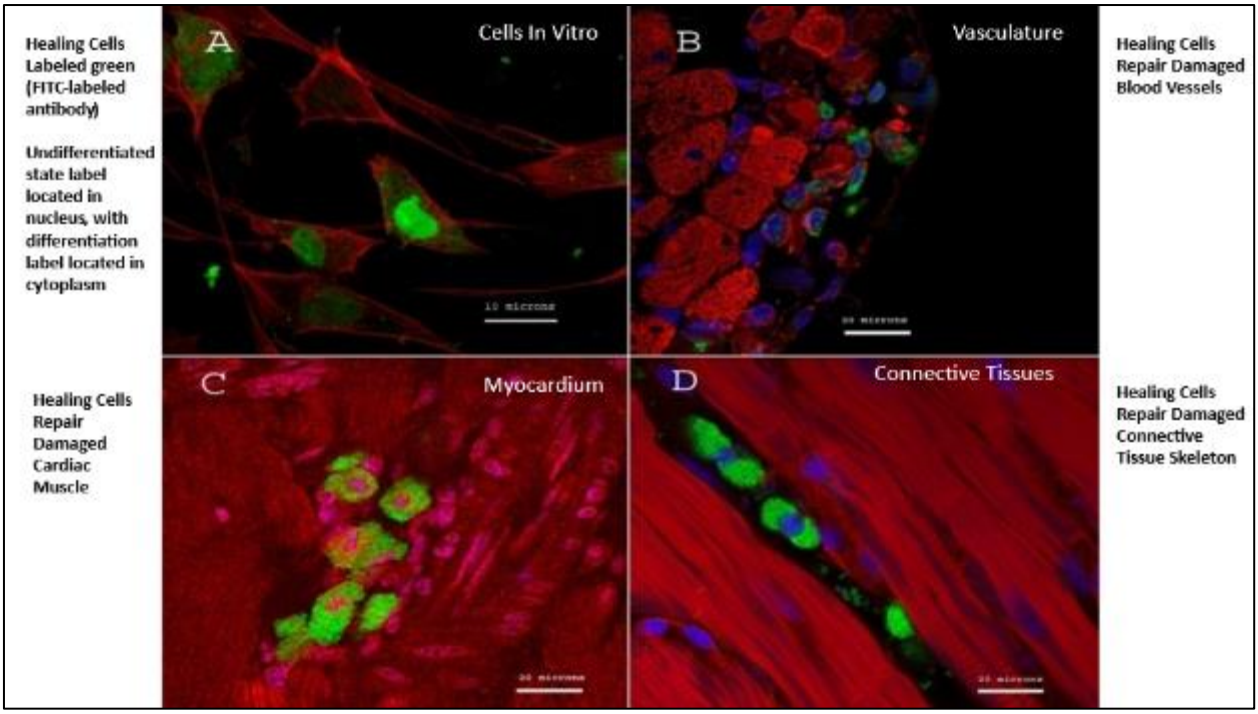


**Figure 6** IACUC-approved Myocardial infarction (apex of left ventricle damaged with liquid nitrogen) animal model in adult Sprague-Dawley rats. Post damage, genomically-labeled cultured clone Scl-40 was injected into damaged site. Six weeks after injection, cultured clone Scl-40 cells and animals were sacrificed and processed for immunocytochemistry using antibody to genomic label (Beta-galactosidase). Fluorochrome label for antibody was fluoresceine isothiocyanate (FITC) (green label). Rhodamine (red) was used to stain myocardium. DAPI (blue) was used to stain nuclei. A. Cultured Scl-40, note location of genomic label in nucleus of undifferentiated cell. B. Cells within the wall of the vasculature express the FITC label. C. Cells within the myocardium express the FITC label. D. Cells within the connective tissue cardiac skeleton express the FITC label. In the above, 6B-D, the Scl-40 clone differentiated into three different cell types within the heart, e.g., vasculature, myocardium, and connective tissue [27]. Reprinted with permission from Young HE, Duplaa C, Romero-Ramos M, Chesselet M-F, Vourc'h P, Yost MJ, Ericson K, Terracio L, Asahara T, Masuda H, Tamura-Ninomiya S, Detmer K, Bray RA, Steele TA, Hixson D, El-Kalay M, Tobin BW, Russ RD, Horst MN, Floyd JA, Henson NL, Hawkins KC, Groom J, Parikh A, Blake L, Bland LJ, Thompson AJ, Kirincich A, Moreau C, Hudson J, Bowyer III FP, Lin TJ, Black Jr AC. Adult reserve stem cells and their potential for tissue engineering. Cell Biochem Biophys, 40(1):1-80, 2004

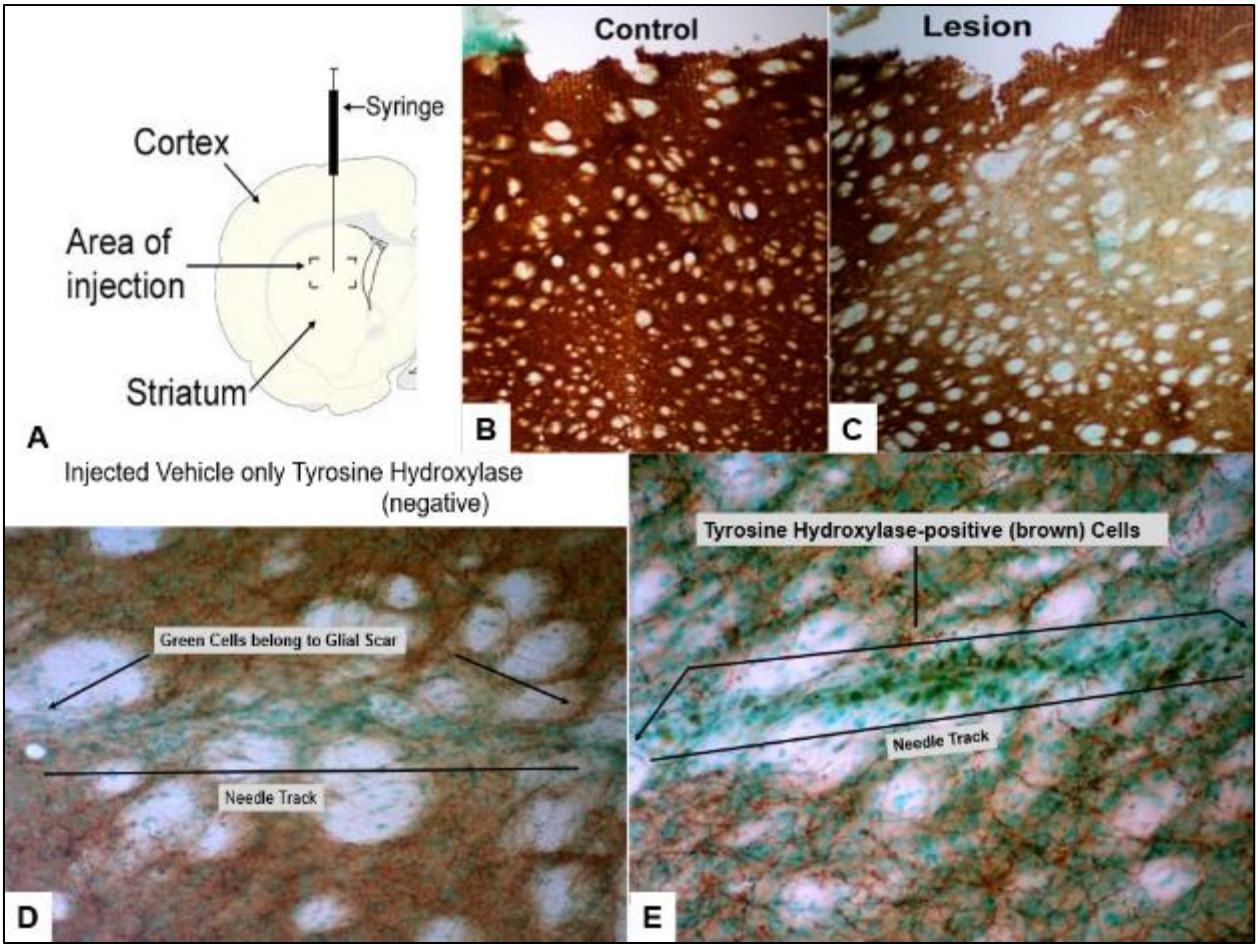


**Figure 7** IACUC-approved animal model for Parkinson's Disease. A. The substantia nigra in the midbrain of outbred Sprague-Dawley rats was lesioned by stereotactic injection of the neurotoxin 6-hydroxydopamine (6-OHDA). Two weeks after injection of 6-OHDA, representative animals (buffer control and 6-OHDA injected experimental) were sacrificed and the brains processed for tyrosine hydroxylase histochemical staining. Tyrosine hydroxylase is the precursor for the synthesis of dopamine B. Buffer-control injection demonstrated no loss of tyrosine hydroxylase activity in the terminus of the injection site. C. In contrast, the side injected with 6-OHDA showed absence of tyrosine hydroxylase staining at the termination site of the 6-OHDA injection. D. 6-hydroxydopamine injected animal (experimental) injected with buffer saline control. Note absence of any tyrosine hydroxylase positive cells along needle track and within adjacent neural networks. In contrast, green cells indicative of glial scarring located along the needle track and within the neural networks. E. 6-hydroxydopamine injected animal (experimental) injected with the pluripotent stem cell clone Scl-40. Note presence of tyrosine hydroxylase positive cells (brown) in needle track as well as within neural networks [42]. Reprinted with permission from Young HE, Duplaa C, Katz R, Thompson T, Hawkins KC, Boev AN, Henson NL, Heaton M, Sood R, Ashley D, Stout C, Morgan JH, Uchakin PN, Rimando M, Long GF, Thomas C, Yoon JI, Park JE, Hunt DJ, Walsh NM, Davis JC, Lightner JE, Hutchings AM, Murphy ML, Boswell E, McAbee JA, Gray BM, Piskurich J, Blake L, Collins JA, Moreau C, Hixson D, Bowyer FP, Black AC Jr. Adult-derived stem cells and their potential for tissue repair and molecular medicine. J Cell Molec Med 2005; 9:753-769

### 3.5. Expected Results

Primitive undifferentiated PSCs, Scl-40, were stereotactically injected into experimental animals previously stereotactically injected with 6-OHDA. Scl-40 formed dopaminergic neurons and dopaminergic neural networks expressing tyrosine hydroxylase enzyme (precursor to dopamine) within the substantia nigra of the mid brain (Fig. 7E) [41].

### 3.6. Unexpected results

Extra injected primitive undifferentiated PSCs, Scl-40, also migrated back along needle track and regenerated normal tissues and cells in the cerebral cortex damaged during both sets of stereotactic injections (6-OHDA and Scl-40).

Undifferentiated PSC clone, Scl-40□, differentiated into cells that also expressed the Beta-Gal label (brown). The cells in the cerebral cortex were glial cells, interneurons, capillary (containing RBCs), and Pyramidal neurons (Fig. 8) [41].

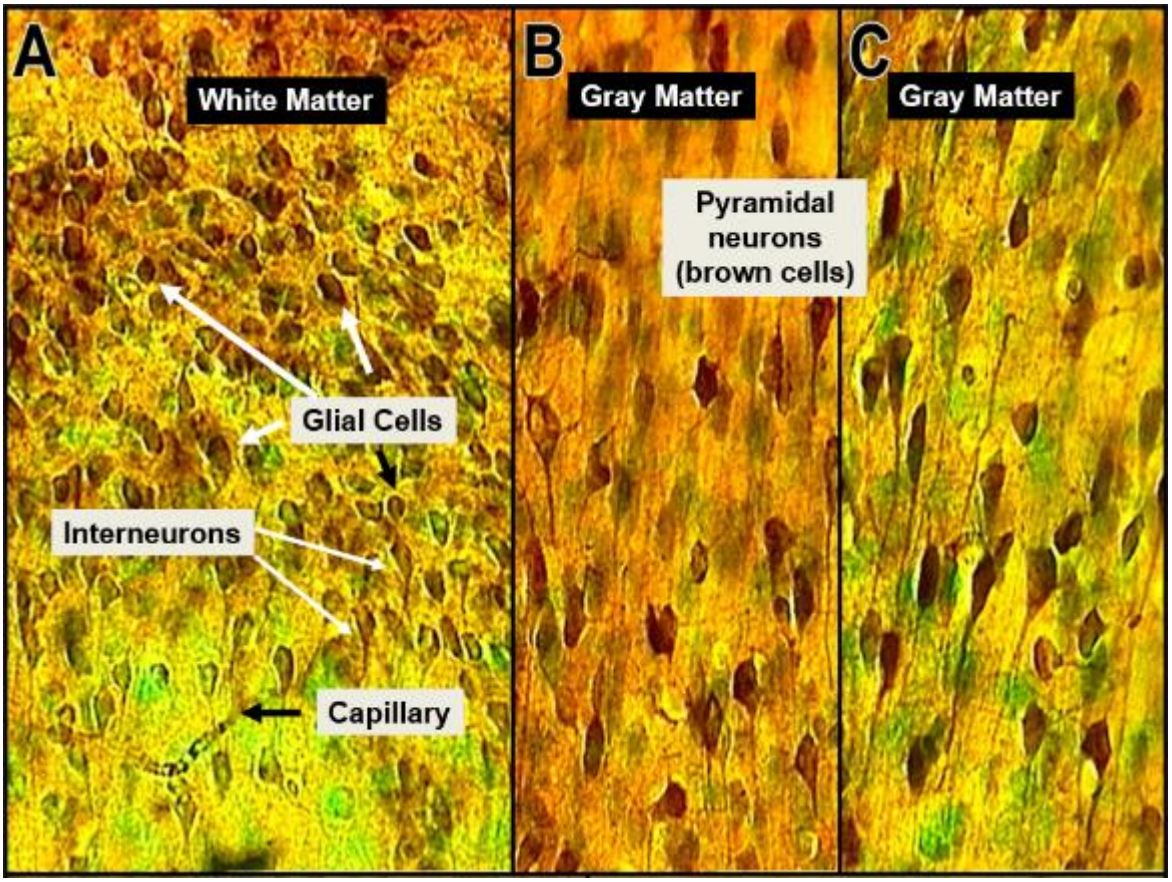


**Figure 8** Cerebral cortex in areas that contained the needle tracks for the 6-hydroxydopamine and the pluripotent stem cell, Scl-40b, injection sites. Beta-Galactosidase expressing cells (brown reaction product) were derived from the injected PSCs. A. Presence of glial cells (brown), interneurons (brown), and endothelial cells (brown) of capillary contained within the cortical white matter. B. & C. Presence of pyramidal neurons (characteristic shape and brown) as well as glial cells (small brown circular entities) within cortical gray matter [41]. Reprinted with permission from Young HE, Duplaa C, Katz R, Thompson T, Hawkins KC, Boev AN, Henson NL, Heaton M, Sood R, Ashley D, Stout C, Morgan JH, Uchakin PN, Rimando M, Long GF, Thomas C, Yoon JI, Park JE, Hunt DJ, Walsh NM, Davis JC, Lightner JE, Hutchings AM, Murphy ML, Boswell E, McAbee JA, Gray BM, Piskurich J, Blake L, Collins JA, Moreau C, Hixson D, Bowyer FP, Black AC Jr. Adult-derived stem cells and their potential for tissue repair and molecular medicine. J Cell Molec Med 2005; 9:753-769

## 4. Discussion

### 4.1. Cell-specific exosome-conditioned medium

When the cloning studies began, we notice that a single cell from any of the categories or subcategories of isolated cells would not survive in complete medium. Two other groups noted the same phenomenon [20,60]. Their response was either to clone using three cells as their starting population with the formation of three cell types, e.g., fat, cartilage, and bone [20]. Or start with ten cells as their starting population with the formation of one cell type from each of the three germ layer lineages [60,61].

However, in studies described herein, it was noted that if conditioned medium from the same cell type was added to the single-cell culture, that the single cell would survive. And if a proliferation agent was then added to the complete medium 1:1 with conditioned medium, the single cell would respond and begin to proliferate. We originally termed components with the conditioned medium as “nearest neighbor phenomenon”, later to be termed exosome-conditioned medium. It was also noticed that the cultures became more robust as the concentration of the conditioned medium increased. A dose-response experiment, e.g., 0x, 1x, 2x, 3x, 4x, 5x, 6x, 7x, 8x, 9x, 10x conditioned medium versus cell

numbers, was performed. The results showed that 5-6x concentrated conditioned medium resulted in an optimal response to both increased cell doublings as well as robustness of the cell cultures. We incorporated 5-6x species-specific cell-specific conditioned medium in the cloning experiments, initially with avian cells [62], then with mouse cells [63], and then rat cells [15].

To obtain cells for cloning, mixed isolates of differentiated cells, progenitor cells, and stem cells were derived from tissue biopsy specimens, plated, and propagated [29]. The cells were then segregated into individual populations, e.g., telomerase negative differentiated cells and progenitor cells versus telomerase positive stem cells, using differential cryopreservation [25]. All three populations (differentiated cells, progenitor cells, and stem cells) were either flash frozen and stored in liquid nitrogen (-196$^{o}$C) [differentiated cells and progenitor cells, MSCs], slow frozen to and stored at both -70$^{o}$C [GLSCs, EctoSCs, MesoSCs, and EndoSCs], or slow frozen and stored at -80$^{o}$C [TSCs, HLSCs, CLSCs, PSCs]. Greater than 95% of either differentiated cells or progenitor cells survived flash freezing and storage in liquid nitrogen (-196$^{o}$C). Greater than 98% of the stem cells survived slow freezing and storage at low temperatures -70$^{o}$C and -80$^{o}$C [25].

Cell sorting with cell-specific cell surface markers, e.g., CEA-CAM-1, SSEA-4, and Thy-1 [25], was utilized to separate the -70$^{o}$C and -80$^{o}$C healing stem cells into separate categories of cells (Tables 3-5). The -80$^{o}$C cells were the TSCs, HLSCs, CLSCs, and PSCs, while the -70$^{o}$C cells were the GLSCs, EctoSCs, MesoSCs, and EndoSCs. The characterization of the cells was initially based on size, cell surface markers, Trypan blue staining, and differentiation potentials [15]. The subcategories were 0.1 to 2.0-micron CEA-CAM-1 positive entities, that were spherical in shape, completely Trypan blue positive, and had the ability to differentiate into all cells of a 4-cell stage blastomere [64]. These cells were termed totipotent stem cells (TSCS). Greater than 2 to 4-micron entities were CEA-CAM-1/SSEA-4 positive that were spherical in shape, had a halo-like rim of Trypan blue staining, and could form any somatic cell of the body, similar to a blastomere from the inner cell mass [64]. Based on the rim of staining, these cells were termed halo-like stem cells (HLSCs). Greater than 4 to less than six-micron entities that were SSEA-4/CEA-CAM-1 positive entities that were spherical in shape and had a crown (corona-like) rim of Trypan blue staining along one side, and had the ability to differentiate into all somatic cells of the body, similar to a blastomere from the inner cell mass [64]. Based on the crown (corona)-like staining pattern, the cells were termed corona-like stem cells (CLSCs). The 6 to 8-micron SSEA-4 positive entities that were Trypan blue negative had the ability to form all somatic cells of the body, similar to a blastomere from the inner cell mass [64]. These cells were termed pluripotent stem cells (PSCs). The 8 to 10-micron entities that were SSEA-4/Thy-1 positive and Trypan blue negative had the potential to form all somatic cells of the body, similar to a blastomere from the inner cell mass [64]. They were termed germ layer lineage stem cells. The 10 to 12 micron Thy-1 positive cells that were also Trypan blue negative could be subdivided into three separate categories based on their respective differentiation potentials. The differentiation capabilities of the three categories of cells noted formation of all somatic cells of either the ectodermal germ layer lineage, termed ectodermal stem cells (EctoSCs), mesodermal germ layer lineage, termed mesodermal stem cells (MesoSCs), or endodermal germ layer lineage, termed endodermal stem cells (EndoSCs) (Table 3).

Within the population of progenitor cells, a tripotent progenitor cell was chosen based on its ability to form three separate cell types, e.g., fat, cartilage, and bone, and was designated as the mesenchymal stem cells (MSCs) [19-21]. Due to the slower doubling time of the MSCs, they did not genomically-label using lipofectin. Clones of genomically-labeled aTPSCs and a non-genomically-labeled tripotent progenitor MSC were used for comparison purposes with respect to subsequent experiments.

The generate cell-specific exosome-conditioned medium, cell sorted mixed cell isolates [25] were plated individually onto either 1% collagen coated culture vessels (stem cells) or bare plastic (progenitor cell, e.g., tripotent mesenchymal stem cell) and used to generate cell-specific exosome-conditioned medium. Avian cells were propagated in Eagle's MEM with Earle's salts with 10% HS7 horse serum, 5% embryo extract, and 1% antibiotic, at pH 7.4. Mouse and rat cells were propagated in OptiMEM + GlutaMax medium containing 10% heat-inactivated serum and 1% antibiotic, at pH 7.4. The Thy-1 positive cells (GLSCs, EctoSCs, MesoSCs, and EndoSCs) were propagated to confluence. The CEA-CAM-1, and/or SSEA-4 positive cells (TSCs, HLSCs, CLSCS, and PSCs) were propagated to multi-layered confluence. The cells were replated into 1% collagen-coated T-75 flasks, and propagated to generate conditioned medium (Fig. 4).

### 4.2. Repetitive single cell clonogenic analysis

For single cell cloning of avian cells 12 x 96-well plates were used for initial plating and two subsequent re-platings for a total of three platings. Plating efficiency was 12% for avian cells, with the generation of 34 clones. For single cell cloning of mouse cells 10 x 96-well plates were used for initial plating and two subsequent re-platings, for a total of three platings. Plating efficiency for mouse cells was 15%, with the generation of 144 clones [63]. For single cell cloning

of rat cells 5 x 96-well plates were used for initial plating and 3 additional re-plating, for a total of four platings. Plating efficiency for rat cells was 20%, with the generation of 96 clones [18-21].

For avian and mouse cells, the resulting clones were characterized until there was a single identifiable clone for each cell type, e.g., e.g., TSCs, HLSCs, CLSCs, PSCs, GLSCs, EctoSCs, MesoSCs, EndoSCs, and MSCs. For rat cells, the resulting clones were characterized until four clones were identified by their characteristic criteria for each of the following cell types, e.g., TSCs, HLSCs, CLSCs, PSCs, GLSCs, EctoSCs, MesoSCs, EndoSCs, and MSCs (Table 5).

### 4.3. Genomic labeling of clones with beta-galactosidase

Rat clones were designated as the recipient of the beta-Galactosidase genomic label. Clones of rat TSCs, HLSCs, CLSCs, PSCs, GLSCs, EctoSCs, MesoSCs, EndoSCs, and MSCs were sent to the INSERM laboratory of Dr. C. Duplaa (France) for genomic labeling using the lipofectin reagent. Lipofectin transfers the genomic label (Lac-Z, an insect gene for Beta-Galactosidase) to the cells during cell division. The more rapidly the cells divide, the greater the efficacy of labeling increases. The highest efficiency of labeling was in the TSCs = HLSCs = CLSCs = PSCs > GLSCs > EctoSCs = MesoSCs = EndoSCs > > > > > > > MSCs. TSCs, HLSCS, CLSCs, and PSCs double every 12-14 hours; GLSCs double every 14-18 hours; EctoSCs, MesoSCs, EndoSCs double every 18-24 hours; and MSCs double from days to weeks (Table 3). The labeling efficacies paralleled the optimum doubling rate of the cells, without forming mutations in the genome [65]. The group of healing stem cells, e.g., EctoSCs, MesoSCs, and EndoSCs, that demonstrated the least labeling efficiency, still recorded a labeling efficacy of >95%. However, the MSC clones that were sent demonstrated a labeling efficiency of 0%.

Fifty-six genomically-labeled clones were received from France. Thus far, five beta-Gal labeled clones have been fully characterized, e.g., Scl-4□ (TSC clone), Scl-9□ (TSC clone), Scl-44□ (TSC clone), Scl-40□ (PSC clone), Scl-$A_2A_2$□ (MesoSC clone). Additionally, four unlabeled rat-derived progenitor cell clones (Rt-MSC, Rt-Adip, Rt-Chon, and Rt-Os) were received. These nine clones (four labeled and four unlabeled) were characterized to ascertain whether the lipofectin procedure altered any of their respective characterization parameters (Table 4) as based on the characterization parameters established previously (Table 3) and [15]. No differences could be detected in any of the labeled clones examined.

## 5. Conclusion

The hypothesis tested was that individual populations of aTPSCs and MSCs could be cloned from single cells.

It was determined that aTPSCs and MSCs could be cloned from single cells if 5-6x conditioned medium from the same cell type was used.

## Compliance with ethical standards

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### *Disclosure of conflict of interest*

No conflict of interest was disclosed.

### *Statement of ethical approval*

**Animal Use** The use of animals in this study complied with the guidelines of Mercer University's Institutional Animal Care and Use Committee (ACUC). These guidelines reflect the criteria for humane animal care of the National Research

Council as outlined in "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health.

*Statement of informed consent*

The use of human biopsy specimens in this study complied with the guidelines of Mercer University's Institutional Review Board (IRB). These guidelines reflect the Federal Regulations for Protection of Human Research Subjects, HHS Office for Human Research Protections – 45 and 46 CFR: 46.102(I).

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